Pre-hybridization and hybridization were done in the manner described in the preceding section 3.11.3.1. Immuno-detection was performed in accordance with the manufacturer's instructions. In this process, bound digoxigenin-labeled DNA is detected using an antibody conjugate (anti-digoxigenin-alkalic-phosphatase conjugate) and a subsequent enzyme color reaction.

When loaded nylon membranes (Hybond N$^+$) were used, it was possible to wash off the bound, labeled DNA again using the method of Gebeyehu et al. (1987); the blots could then be reused.

4. Results

4.1 Changes in the genome of the vaccinia virus strain Ankara during the course of passaging

4.1.1 Characterization of the DNA of plaque isolates of initial strain CVA 2

A number of plaques of the initial strain CVA 2 were examined in connection with plaque cleaning. Figure 1 shows their DNA following cutting with the restriction endonuclease HindIII. Three variants (tracks 3, 5, 7) were compared with the non-cloned initial strain CVA 2 (track 1). In comparison to the plaque isolates, the initial strain features a few submolar bands in the region of the large fragments. A few of these submolar bands, different in each case, prove to be unimolar in the plaque isolates. Because we assumed that there would be length variation among the terminal fragments, a "cross link" preparation was used for identification of the terminal fragments in tracks 2, 4, 6 and 8. The terminal fragments are clearly recognizable in the plaque isolates: 38 and 27 Kbp for variant I, a bimolar 13 Kbp fragment for variant II and a 13 and a 12 Kbp terminal fragment for variant III. These are only poorly visible in the non-cloned initial strain, due to the many submolar terminal fragments.

The genome terminal fragments of the sub-populations (I, II, II) of CVA 2 following cutting with restriction endonuclease XhoI are shown in magnified form in Figure 2. The results confirm the changes observed following HindIII digestion: The genome end attenuates bilaterally by 1 Kbp from variant I to variant II, while variant III, in comparison to variant II, has lost an additional 1 Kbp at the left genome end.

Because the section pattern of variant I was most clearly recognizable in the non-cloned material, and because this variant was also isolated most frequently during plaque cleaning, this strain, following two additional plaque cleanings, was used as the initial material for subsequent experiments.

Figure 1

Figure 2

4.1.2 Characterization of the DNA of various passages of the vaccinia virus strain Ankara with the aid of restriction endonucleases

Figures 3, 4 and 5 provide an overview of the size of the DNA fragments of various passages of the vaccinia virus strain Ankara following digestion with the restriction enzymes HindIII and XhoI.

Following digestion with HindIII and separation of the DNA fragments in a 0.5% agarose gel (Figure 3), a rather uniform image is obtained, due to the constant drift behavior of most fragments. To facilitate classification, the DNA bands are labeled alphabetically from top to bottom, arranged according to their molecular weights. In the upper gel region, the three largest DNA fragments, A, B and C, differ in their drift behavior. The molecular weight of each band gradually decreases from CVA 2 through CVA 382 to MVA 574.

To depict the smallest HindIII fragments of CVA 2 and MVA 574, these fragments were separated in a 1.2% agarose gel (Figure 4). In the molecular weight range of 0.5 to 3 Kbp, four DNA bands are visible in the section pattern of CVA 2, while only two fragments are visible in that of MVA 574. This variation in the HindIII pattern image already occurred during the passaging from CVA 2 to CVA 382, with no further change subsequently occurring within this size range.

In contrast, the XhoI section pattern of the DNA of CVA 2, CVA 382, MVA 574 and CVA ens is significantly more varied (Figure 5). There are only a few constantly occurring bands. The number of XhoI fragments obtained declines from CVA 2 (18 bands) to MVA 574 (14 bands). In contrast to the HindIII section pattern, a few bands appear in double or triple molar form, due to higher intensity.

Figure 3

Figure 4

Figure 5

4.13 Determination of the genome size of various passages of the vaccinia virus Ankara

For precise determination of size, DNA fragments with a molecular weight greater than 20 Kbp were isolated from the agarose gel and sub-digested with a second restriction endonuclease. The total size could then be determined much more precisely, using the molecular weight standards, by summation of the sub-fragments (Tables 2, 3). The addition of the molecular weights of all HindIII fragment revealed that the total genome length, during the course of passaging on HEF cell cultures, declined from 208,000 base pairs for CVA 2, to 188,000 base pairs for CVA 382, and to 177,000 base pairs for MVA 574. This corresponds to a loss of 15% of the

total genome. The total length of the genome of CVA ens comprises 202,000 base pairs. The total genome lengths obtained by summation of the XhoI fragments confirm the measured genome sizes. Tables 2 and 3 show the fragment sizes (in Kbp) for the vaccinia virus DNA cut with HindIII and XhoI, as well as the corresponding total genome sizes.

Table 2

Table 3

Table 4

Table 5

4.2 Depiction of genome changes during passaging

4.2.1 Identification of the terminal fragments

Fragments B and C could be identified as terminal fragments in the HindIII section pattern of the genome of CVA 2, CVA 382, MVA 574 and CVA ens (Figure 11).

The size of the two fragments decreases significantly from CVA 2 through CVA 382 to MVA 574 during the course of passaging (Table 4).

Following digestion with enzyme XhoI, only one fragment could be depicted as a terminal fragment in CVA 2, CVA ens and vaccinia virus Elstree. However, the corresponding DNA band is visible in the agarose gel as a bimolar band in the molecular weight range of 9.6 and 10.0 and/or 7.9 Kbp. In CVA 382 and MVA 574, this symmetry of the genome end is not preserved; the XhoI terminal fragments are 18.4 and 14.8 Kbp in size (Figure 12).

4.2.2 Physical mapping of the vaccinia virus strain Ankara

The internationally recognized physical gene cards for the vaccinia virus strains Elstree and Western Reserve (Mackett and Archard, 1979) were used as the basis for the development of physical gene cards for passages CVA 2, CVA 382, MVA 574 and CVA ens. This project was facilitated for the HindIII card by the fact that the drift behavior of most fragments was similar.

Because only a few fragments occurred constantly, and despite the fact that gene cards already existed, direct assignment was not possible following digestion with the restriction endonuclease XhoI. However, the fluctuating band pattern was well-suited for the localization of genome changes in hybridizations.

The mapping of the genome ends was complicated by the bilateral ITR regions in the virus genome, because terminal fragments hybridized with one another alternately. To map the virus genome, XhoI DNA fragments were cross-hybridized on the HindIII fragment pattern and, conversely, HindIII fragments were cross-hybridized on the XhoI section pattern of the vaccinia virus passages (Table 6). During cross-hybridization, the large HindIII terminal fragments B and C of CVA 2 both reacted with the XhoI fragments H, K, E and G of CVA 2 and the XhoI fragments D and E of CVA 382. In addition, the HindIII B fragment recognized the XhoI B fragment of CVA 2 and the XhoI C fragment of CVA 382, while the HindIII C fragment of CVA 2 also hybridized with the XhoI D fragment of CVA 2 and the XhoI fragments A and H of CVA 382. The XhoI H

fragment of CVA 382 proved to be suitable for identifying the HindIII C fragment of the vaccinia virus Ankara as a left-sided terminal fragment corresponding to the already mapped HindIII B fragment of vaccinia Elstree. Through hybridization with the XhoI A fragment of MVA 574, the HindIII fragments of the left genome side could be depicted into the constant central genome region. The HindIII A fragment of CVA 2 recognized the corresponding XhoI fragments of the right genome side. Fragments that behaved constantly in comparison to the existing gene cards of vaccinia virus were not included in the hybridization experiments.

The SmaI interface in the genome of CVA 2 could be localized, by double digestion with the enzymes SmaI and XhoI, in the XhoI B fragment of CVA 2. In this context, an interface for SmaI in the XhoI A fragment, which had not been described previously, was found in the genome of vaccinia Elstree.

The physical cards of the virus genomes from the enzymes HindIII, XhoI and SmaI demonstrate that deletions occurred in the left and right halves of the genome during the attenuation of CVA 2 to MVA 574 (Figures 7, 8) (also see section 4.3). In contrast, a comparison of the gene cards of CVA 2 and CVA ens reveals only minor changes (Figure 9).

Table 6

Figure 7

Figure 8

Figure 9

4.3 Characterization of the changes in the genome occurring during virus passaging

Several DNA segments were lost during the course of passaging from CVA 2 to MVA 574. Through restriction enzyme analysis and hybridization, at least 6 deletions could be determined and localized in the physical card. The deletions are referred to in the following as I to VI. In this connection, progressing from the left to the right end of the genome, deletions I to IV, which occurred during passaging from CVA 2 to CVA 382, are described first, followed by deletions V and VI from the passage period from CVA 382 to MVA 574.

Deletion I:
The shrinking of the HindIII C fragment of CVA 382 is also apparent upon comparison of the HindIII section image of the DNA of CVA 2 and CVA 382 in the agarose gel (Figure 3). The DNA loss is confirmed by sub-digestion of the HindIII C fragments of CVA 2 and CVA 382 with the enzyme XhoI. The summation of the sizes of the sub-fragments obtained reveals an attenuation of the HindIII C fragment of CVA 382 by 2.8 Kbp (Table 2). In hybridizations, the XhoI G fragment of CVA 2 reacted with the Xho fragments E and H of CVA 3382 and the XhoI fragments A and F of MVA 574. Because, conversely, the Xho fragment H of CVA 382 only hybridizes with the XhoI fragments G of CVA 2 and A of MVA 574, a deletion can be localized in the region of the XhoI G fragment of CVA 2 (Figure 10). Addition of the sizes of the corresponding XhoI fragments resulted in the deletion of 2.9 Kbp.

Deletion II:
When the HindIII fragments of CVA 2 and CVA 382 are considered, it becomes apparent that two fragments sized 2.2 Kbp and 1.5 Kbp are missing in the DNA pattern of CVA 382; in return, a new, 1 Kbp fragment appears (Figure 4). In hybridization experiments, this new fragment N of CVA 382 reacted with the HindIII fragments M and N of CVA 2 missing in CVA 382. Both fragments only hybridized with the N fragment of CVA 382 and/or MVA 574 (Figure 11). As a result of the deletion of a 2.7 Kbp DNA segment, a HindIII interface in the genome of CVA 382 was lost. The molecular weight comparison of the XhoI fragments D and A

of CVA 2 with the XhoI A fragment of CVA 382 confirms a corresponding DNA loss in this genome region (Table 3).

Deletion III:
An attenuation of the HindIII A fragment of CVA 382 in comparison to CVA 2 is difficult to detect in the agarose gel. A 3.3 Kbp deletion in the region of the XhoI B fragment of CVA 2 result only occurred during the sub-digestion of this largest HindIII fragment with the XhoI enzyme (Table 2). In the hybridization experiment, the XhoI B fragment of CVA 2 only reacted with the XhoI C fragment of CVA 382 (Figure 12). The difference in size between these two fragments was 4.2 Kbp. Because a HindIII interface is missing at the transition between the HindIII A fragment and the HindIII B fragment in the genome of CVA 382, and because the corresponding 0.7 Kbp HindIII P fragment of CVA 2 does not hybridize with any fragment of CVA 382, a deletion size of 4.0 Kbp could be computed for the HindIII card. The interface for the enzyme SmaI, which was lost in the genome of CVA 382, also lies in this deletion region.

Deletion IV:
The significant shrinking of the HindIII B fragment of CVA 382 is already apparent upon consideration of the HindIII terminal fragments of the virus genome. A comparison of the sizes of the HindIII B fragments of CVA 2 and CVA 382 by undercutting with XhoI confirms a DNA loss on the order of 10.9 Kbp (Table 2). The XhoI fragments E, K and H of CVA 2, located on the corresponding genome, all hybridize with the XhoI terminal fragment of CVA 382 (Figure 13). This results in the deletion of a 10.2 Kbp DNA segment, which corresponds to the loss of two XhoI segments.

Deletion V:
The depiction of the HindIII section pattern shows that the HindIII C fragment of the left end of the genome was attenuated once again during the passage period from CVA 382 to MVA 574 (Figure 3). During hybridization, the XhoI A fragment of MVA 574 reacted with fragments A and H of CVA 382; conversely, the XhoI H fragment of CVA 382 only hybridized with the XhoI A fragment of MVA 574 (Figure 14). As a result of the loss of an XhoI interface,

the XhoI A fragment of MVA 574 was enlarged. However, an exact comparison of the sizes of the DNA fragments, taking into account the size of the XhoI H fragment of CVA 382, resulted in a DNA loss of 4.7 Kbp in this genome region. The summation of the sub-fragments of the HindIII C fragments of CVA 382 and MVA 574 confirmed a deletion of 4.6 Kbp (Table 2).

Deletion VI:
The attenuation of the HindIII A fragment of MVA 574, as compared with that of CVA 382, can only be presumed in the agarose gel. In cross-hybridizations, it was possible to demonstrate that the XhoI B fragment of MVA 574 is completely contained in the HindIII A fragment (Table 6). The XhoI B fragment of MVA 574 hybridized with the XhoI fragments B and F of CVA 382; conversely, the XhoI F fragment of CVA 382 reacted with the XhoI B fragment of MVA 574 (Figure 15). Here, the enlargement of the XhoI B fragment of MVA 574 as a result of the loss of an XhoI interface also encompasses a net deletion of 6.2 Kbp. When the molecular weight of the HindIII A fragments of MVA 574 and CVA 382 was examined by means of sub-digestion with XhoI, it was possible to calculate a DNA loss of 6.2 Kbp in the genome of MVA 574 (Table 2).

Figure 10

Figure 11

Figure 12

Figure 13

Figure 14

Figure 15

4.4 Marker rescue of the vaccinia virus hose range gene

In transfection experiments with the plasmid pT% 5.2, the 2.7 Kbp deletion (II) in the "host range" gene region in the left half of the genome of MVA 574 and CVA 382 was to be replaced. Following pre-infection with MVA 574 and/or CVA 382 and addition of he $CaCl_2$ DNA precipitate, cell densification occurred 2-4 days p.i., forming central holes. Only virus-infected E-Derm cell cultures, which exhibited no cytopathic effect (CPE), even after five blind passages, were used as negative controls. In addition, $CaCl_2$ precipitated plasmid DNA without insert was also used a negative control. This DNA also exhibited no CPE after five blind passages.

The restriction enzyme analyses of the genomes of the constructs #MVA and #CVA 382 isolated from the marker rescue experiments confirmed the anticipated inclusion of the cloned DNA insert of CVA 2 in the genome of CVA 382 and MVA 574. This is shown in Figure 16, in which the HindIII section patterns of the DNA of MVA 574 and #MVA are compared. The separation of the fragments was done in a chronologically staggered manner in a 0.8% agarose gel. The large DNA fragments of MVA 574 and #MVA following 36 hours of electrophoresis are shown in tracks 2 and 3. Tracks 4 and 5 show the small HindIII bands of the same DNA preparation. The genomes of MVA 574 and #MVA differ only in the region of the small HindIII fragments. In the section pattern of the DNA of #MVA, the 0.1 bp fragment M of MVA 574 has disappeared; in return, two new DNA fragments sized 1.5 Kbp and 2.2 Kbp are visible. These fragments correspond to the DNA bands M and N of CVA 2, which lie in the region of the deletion to be replaced. Examination of the DNA of #MVA and MVA 574 using the restriction enzyme XhoI was not well-suited for confirmation of the inclusion of the DNA insert, because the deletion (II) lies in the region of the large XhoI A fragment. In the XhoI section pattern, a difference in the sizes of the A fragments of #MVA and MVA 574 could only be surmised; otherwise, however, no visible changes in the DNA structure occurred here. Thus, the cloned EcoRI insert of CVA 2 had only inserted itself into the genome of MVA 574 at the expected locus, replacing the deletion which had occurred there.

Figure 16

4.5 Depiction of viral proteins

4.5.1 PAGE gel and immunoblot

The proteins of CVA 2, CVA 382, MVA 574 and Elstree separated in the PAGE gel exhibit an essentially uniform appearance following silver coloration; the band patterns of MVA 574 and CVA 382

are identical (Figure 17A). The minor difference in the size of a polypeptide is apparent in the molecular weight range of 35-38 KD. The protein appears as a 37 KD band in CVA 1 and Elstree, and, in contrast, as a 36 KD band in CVA 382 and MVA 574. The constructs #MVA and #CVA behave in the same manner as MVA 574 and CVA 382, but are not shown in Figure 17.

Essentially three polypeptides, at 58, 32 and 30 KD, appear as primary immunogenic components in the immunoblot process (Figure 17B). However, the difference in size of the 36/37 polypeptide is detectable. Other slightly immunogenic bands react in the same manner.

A 39 KB protein, which only appears in CVA 2 but is not detectable in silver coloration, reacts in the immunoblot. The MAK 5B4, which recognizes an epitope of the virus surface, reacts with a 30 KD protein band with all other viruses. It is identical with the smallest primary immunogenic band detected by the hyperimmune serums.

Figure 17

4.5.2 Reaction with monoclonal antibodies

During the examination in the diagnostic ELISA for differentiation of orthopox viruses, the reaction pattern of the 9 monoclonal antibodies exhibits no differences between MVA 574 and #MVA and/or among CVA 2, CVA 382 and #CVA. However, MVA 574 and #MVA can be differentiated from the initial strain CVA 2 and the 382nd passage, as well as from the construct #CVA. In Figure 18, it is apparent that the strains MVA 574 and #MVA bind only weakly with the MAK 2, 3 and 4 (extinction values < 0.2); however, they react significantly with CVA 2, CVA 382 and #CVA 382 (extinction values >0.8).

Figure 18

4.6 Behavior in the cell culture

4.6.1 Cytopathic effect (CPE)

In the case of C2, the CPE on HEF cells is characterized by the image of crumbly degeneration. The cell bond swells up and fuses into a granulated mass. Holes appear in the cell lawn, and the subsequent lysis is reflected in disintegration into polymorphous, highly granulated cell components. In contrast, the CPE in the case of CVA 382 and MVA 574 is characterized by balling and substantial granulation of the infection cells. Cell fusion or syncytium development do not occur. The constructs #MVA and #CVA exhibit similar behavior. In this case, however, giant cells also occur.

The initial strain CVA 2 replicates on the monkey kidney cell line MA 104, forming large, lytic plaques with uniform balling of the infected cells. Following brief adaptation, CVA 382 and MVA 574 also exhibit a pronounced CPE, but with significantly smaller plaques, balling and syncytium development. The constructs #MVA and #CVA 382 grow on MA 104 cells, forming lytic plaque; instead of balling, the focus here is on syncytium development and cell fusion.

CVA 2 grows on the cell lines RK 13 and E-DERM, rapidly forming lytic plaques and cell balling. In the case of CVA 382 and MVA 574, no visible changes occur on these cell lines. In contrast, the CPE of #MVA and #CVA 382 is characterized by a proliferative effect, which is expressed in the appearance of densification zones and the accumulation of balled-off cells. Only after central lysis do plaque holes finally develop in the cell lawn.

Table 7

4.6.2 Host range

The replication capacity of CVA 2, MVA 574, #MVA and #CVA 382 was determined, following culturing on 14 different cell cultures, by reverse titration on chicken embryo fibroblasts (Table 8; Figure 19). The ratio of the virus titer, after 72 hours, to the titer of the absorbed virus at hour 0 clearly demonstrates the receptiveness of the HEF cells. Titer increases of 3-4 powers of ten occur for all virus strains.

No replication occurs on cell lines MDBK and DBT. After 72 hours, the titer is exactly as high or low as it is following virus adsorption. The ratio is equal to or less than 1. While CVA 2 exhibited a titer increase by a factor of 100-3,000 on all other cell lines, MVA 574 was only capable of effectively replicating on the chicken fibroblast cultures (HEF, LSCC) and on the monkey kidney cell line MA 104, but only replicated weakly on human embryonic lung cells (HEL).

The constructs #MVA and #CVA 382 assume a moderate position in terms of their growth behavior. As expected, they exhibited good replication capability on HEF and MA 104 cell cultures; in contrast to MVA 574, however, they exhibited very good growth on E-DERM and RK 13 cells. In addition, #MVA and #CVA 382 could also be replicated on the human cell line HEP 2 and on the monkey cell line Vero, although they do not attain the same degree of growth here as the initial strain CVA 2. The various types of replication of CVA 2, MVA 574 #MVA and #CVA 382 on permissive, semi-permissive and non-permissive cell lines were summarized in the depiction of comparative growth curves (Figure 19).

Table 8